

## Directed differentiation of pluripotent cells to neural lineages: homogeneous formation and differentiation of a neurectoderm population

Joy Rathjen<sup>1</sup>, Bryan P. Haines<sup>1,\*</sup>, Kathryn M. Hudson<sup>1</sup>, Antonietta Nesci<sup>2</sup>, Stephanie Dunn<sup>2</sup> and Peter D. Rathjen<sup>1,3,†</sup>

<sup>1</sup>Department of Molecular Biosciences, <sup>2</sup>Cell Therapy Program and <sup>3</sup>ARC SRC for the Molecular Genetics of Development, The University of Adelaide, Adelaide, South Australia 5005, Australia

\*Present address: Section of Gene Function and Regulation, Chester Beatty Laboratories, Institute of Cancer Research, London SW3 6JB, UK

†Author for correspondence (e-mail: peter.rathjen@adelaide.edu.au)

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### SUMMARY

During embryogenesis the central and peripheral nervous systems arise from a neural precursor population, neurectoderm, formed during gastrulation. We demonstrate the differentiation of mouse embryonic stem cells to neurectoderm in culture, in a manner which recapitulates embryogenesis, with the sequential and homogeneous formation of primitive ectoderm, neural plate and neural tube. Formation of neurectoderm occurs in the absence of extraembryonic endoderm or mesoderm and results in a stratified epithelium of cells with

morphology, gene expression and differentiation potential consistent with positionally unspecified neural tube. Differentiation of this population to homogeneous populations of neural crest or glia was also achieved. Neurectoderm formation in culture allows elucidation of signals involved in neural specification and generation of implantable cell populations for therapeutic use.

Key words: Stem cells, Neurectoderm, Cell culture, Neural crest

### INTRODUCTION

Cell therapy, the use of cells for the correction of disease, provides an opportunity to create therapeutic agents for previously untreatable human diseases caused by cell damage or dysfunction. Potential sources of therapeutic cells include the differentiated products of human adult stem cells, such as neural stem cells and haemopoietic stem cells, and human pluripotent cells representative of pluripotent cells of the early embryo (Rathjen et al., 1998). In order to fulfil requirements for cell production, progenitor cells must proliferate in culture, preferably from clonal isolates, allow precise genetic manipulation of the genome and differentiate effectively to cell populations suitable for implantation.

The best characterised pluripotent cells are mouse embryonic stem (ES) cells isolated from the inner cell mass (ICM) of the preimplantation blastocyst (Evans and Kaufman, 1981; Martin, 1981; Brook and Gardner, 1997). ES cells can be maintained stably as a pluripotent cell population in culture for indefinite periods of time in the presence of gp130 agonists and support both clonal proliferation and precision genome modification (reviewed by Smith, 1992; Rathjen and Rathjen, 2001). Withdrawal of gp130 signalling, formation of embryoid bodies (EBs) or reintroduction to the early mouse embryo leads to differentiation of ES cells into a variety of differentiated cell populations, which can include all embryonic and adult cell populations, including the germ lineage (Bradley et al., 1984; Doetschman et al., 1985). Mouse

ES cells therefore fulfil the requirements for cell therapy applications, although methodologies for controlled differentiation of these cells have not been described. Human ES cells, with similar properties to mouse ES cells, have been reported (Thomson et al., 1998; Reubinoff et al., 2000) but not yet characterised extensively.

ES cells can be aggregated and differentiated in suspension culture. In the absence of gp130 signalling, aggregated ES cells form structures termed embryoid bodies (EBs), which recapitulate many aspects of cell differentiation during early mammalian embryogenesis (Doetschman et al., 1985; Shen and Leder, 1992; Lake et al., 2000). Outer cells form extraembryonic endoderm and its derivatives while inner cells undergo processes equivalent to formation of the proamniotic cavity (Coucovanis and Martin, 1995) and primitive ectoderm (Shen and Leder, 1992; Lake et al., 2000), followed by pluripotent cell differentiation into differentiated tissues derived from all three germ layers. EB differentiation potentially provides a model system for the characterisation of early embryonic events and a protocol for the formation of therapeutically useful cell populations. However, the lack of structural organisation and positional information within EBs during pluripotent cell differentiation results in heterogeneity both within and between EBs, and exposure of differentiating cells to potentially inappropriate signalling environments resulting from the juxtaposition of temporally and spatially distinct cell populations (Rathjen and Rathjen, 2001). This potentially limits the use of EBs as a model system of early

mammalian embryogenesis and for the production of cell populations with therapeutic application.

Lineage-specific differentiation of ES cells to both primitive ectoderm and subsequently mesoderm has been achieved by manipulation of the differentiation environment. ES cells cultured as monolayers in the presence of medium conditioned by the human hepatocellular carcinoma cell line HepG2 (MEDII) have been shown to form a second, stable pluripotent cell population, early primitive ectoderm-like EPL cells (Rathjen et al., 1999). EPL cells demonstrate morphology, gene expression, differentiation potential and cytokine responsiveness distinct from ES cells but characteristic of the post-implantation pluripotent cell population of the mouse embryo, primitive ectoderm. Further differentiation of EPL cells within EBs results in the efficient formation of mesoderm at the expense of both visceral endoderm and embryonic ectodermal lineages (Lake et al., 2000).

While formation of populations enriched in neural cells, from ES cells, has been achieved by differentiation in the presence of retinoic acid (Bain et al., 1996), use of selective medium on ES cells and the products of EB differentiation (Okabe et al., 1996; Tropepe et al., 2001), coculture with inactivated feeder layers (Kawasaki et al., 2000) or use of genetically modified ES cells and antibiotic selection to select for cells expressing early neural markers (Li et al., 1998), there are inherent deficiencies in these approaches (Rathjen and Rathjen, 2001). For example, neural precursors produced in response to retinoic acid induction appear to be developmentally restricted such that further differentiation results in production of a limited range of neural cell types (Renoncourt et al., 1998). Furthermore, formation of neural progenitors in the presence of other cell lineages, as generated for example within EBs, may expose developmentally plastic cells to inappropriate signals. Finally, selective techniques are limited to cells with specific properties, such as gene expression or survival, which may not necessarily be those best suited to further analysis or exploitation.

Genetic and biochemical analysis of neurectoderm specification, patterning and differentiation, and production of cells for therapeutic application, would be facilitated by the availability of an embryologically relevant population of neural precursors generated by stepwise, homogeneous differentiation of ES cells in a manner recapitulating establishment of this lineage during embryogenesis. Here, we describe a novel approach to generation of neural lineages, via directed differentiation of ES cells to a homogeneous population equivalent to embryonic neurectoderm without the formation of embryoid bodies, extraembryonic cell populations or other germ lineages and without the use of selective techniques. Differentiation of ES cells in suspension in medium supplemented with MEDII resulted in recapitulation of neurectoderm formation in the embryo with the ordered and synchronous appearance of primitive ectoderm, neural plate and neural tube equivalent populations. The resulting neurectoderm population comprised a columnar epithelial sheet, consistent with the morphology of this cell population *in vivo*, which expressed early neural markers but did not express genes associated with positional specification. Homogeneous differentiation of pluripotent cell-derived neurectoderm to neural crest or glia, and the demonstration of neuron formation, was consistent with an unrestricted

differentiation potential and provided the first demonstration of directed terminal differentiation of pluripotent cells in culture. Recapitulation of formation of the mammalian neural lineage *in vitro*, in the absence of potentially instructive signals originating from other cell lineages, provides a system for evaluation, at a molecular and cellular level, of the mechanisms of neurectoderm formation.

## MATERIALS AND METHODS

### Cell culture

ES cell lines E14 (Hooper et al., 1987) and D3 (Doetschman et al., 1985) were used in this study. Routine culture of ES and EPL cells and production of MEDII- and sfMEDII-conditioned medium were as described by Rathjen et al. (Rathjen et al., 1999). Briefly, HepG2 cells (Knowles et al., 1980) (ATCC HB-8065) were trypsinised to a single cell or near single cell suspension and seeded at  $5 \times 10^4$  cells/cm<sup>2</sup> in DMEM (Gibco BRL #12800) supplemented with 10% foetal calf serum (FCS; Commonwealth Serum Laboratories) and 1 mM L-glutamine to give a ratio of  $1.75 \times 10^5$  cells/ml medium. Conditioned medium was collected after 4 days culture, sterilised by filtration through a 22 µm membrane and supplemented with 0.1 mM β-mercaptoethanol (β-ME) before use. MEDII was stored at 4°C for 1–2 weeks. For these experiments MEDII was not frozen. HepG2 cells were replenished from frozen stocks every 2 months.

### Formation of cell aggregates

All cell aggregates were formed from single cell suspensions ( $1 \times 10^5$  cells/ml) of ES or EPL cells cultured in bacterial Petri dishes. ES cell and EPL cell embryoid bodies (EBs and EPLEBs respectively) were formed as described previously (Lake et al., 2000). EBMs, cell aggregates formed and maintained in MEDII, were formed from ES cells aggregated in IC:DMEM (DMEM with 10% FCS, 40 mg/ml gentamicin, 1 mM L-glutamine and 0.1 mM β-ME) supplemented with 50% MEDII. Aggregates were divided 1 in 2 on days 2 and 4, and medium was changed on days 2 and 4 and then daily until collection. In early experiments 10–20 ng/ml FGF4 was added to the medium from day 4, however this did not influence the outcome of differentiation and was omitted in later experiments. The time in days from formation of aggregates was denoted by superscript with the day of formation denoted as day 0. For example, EBM 5 days after formation are represented as EBM<sup>5</sup>.

For continued suspension culture of EBs and EBMs, aggregates on day 7 were transferred to serum-free medium (50% DMEM, 50% Hams F12; Gibco BRL # 11765) supplemented with 1× insulin-transferrin-sodium selenite (ITSS) supplement (Boehringer Mannheim) and 10 ng/ml FGF2 (Peprotech).

For adherent culture, aggregates were seeded onto gelatin-treated tissue-culture grade plasticware (Falcon) on day 7 of development in 500 µl DMEM supplemented with 10% FCS (Commonwealth serum Laboratories). On day 8 medium was removed and replaced with 50% DMEM, 50% Hams F12 supplemented with 1× ITSS (Boehringer Mannheim).

### Analysis of differentiation potential of cells within cellular aggregates

EB<sup>7</sup> and EBM<sup>7</sup> were seeded as described above and assessed on days 8, 10, 12 and 14 for the presence of neurons, identified morphologically by the presence of axonal projections (and confirmed by the expression of NF200; data not shown), and beating cardiocytes, identified morphologically by rhythmical contraction of cells within the aggregate.

### Neural crest formation

EBM<sup>9</sup> were collected, washed in PBS, treated with 0.5 mM EGTA

pH 7.5 for 3 minutes, washed in PBS and disaggregated to small clumps (20-200 cells) by trituration. Cell clumps were allowed to settle and single cells liberated during trituration were removed with the supernatant before plating onto tissue-culture grade plasticware that had been coated with cellular fibronectin (1  $\mu\text{g}/\text{cm}^2$ ; a gift from M. D. Bettess, Department of Biochemistry, Adelaide University, Australia) and allowed to dry. Cells were cultured in Hams F12 containing 3% FCS and 10 ng/ml FGF2 and supplemented with either 0.1% 25  $\mu\text{M}$  staurosporine (Sigma) in DMSO (final concentration, 25 nM) or 0.1% DMSO. Cellular aggregates were allowed to differentiate for 48 hours before fixation in 4% paraformaldehyde (PFA) for 30 minutes.

### Glial lineage formation

EBM<sup>9</sup> were collected, washed in PBS and broken into small clumps as described above. Cell clumps were transferred to tissue culture plasticware pretreated with poly-L-ornithine as per the manufacturer's instructions (Sigma) and cultured in 50% DMEM, 50% F12, 1 $\times$  ITSS, 1 $\times$  N2 supplement (Sigma), 10 ng/ml FGF2, 20 ng/ml EGF (R&D Systems Inc.) and 1  $\mu\text{g}/\text{ml}$  laminin (Sigma). Medium was changed daily. After 5 days medium was changed to 50% DMEM, 50% F12, 1 $\times$  ITSS, 1 $\times$  N2 supplement (Sigma), 10 ng/ml FGF2 and 10 ng/ml PDGF-AA (R&D Systems Inc.). Cells were fixed for analysis on day 7 or 8 of culture by treatment with 4% PFA for 30 minutes.

### Gene expression analysis

#### Northern blot analysis

Cytoplasmic RNA was isolated from cellular aggregates using the method of Rathjen et al. (Rathjen et al., 2001). Northern blot analysis was performed as described previously (Thomas et al., 1995). DNA probes were prepared from DNA fragments using a Gigaprime labelling kit (Bresagen). DNA fragments used were as described previously (Rathjen et al., 1999; Lake et al., 2000).

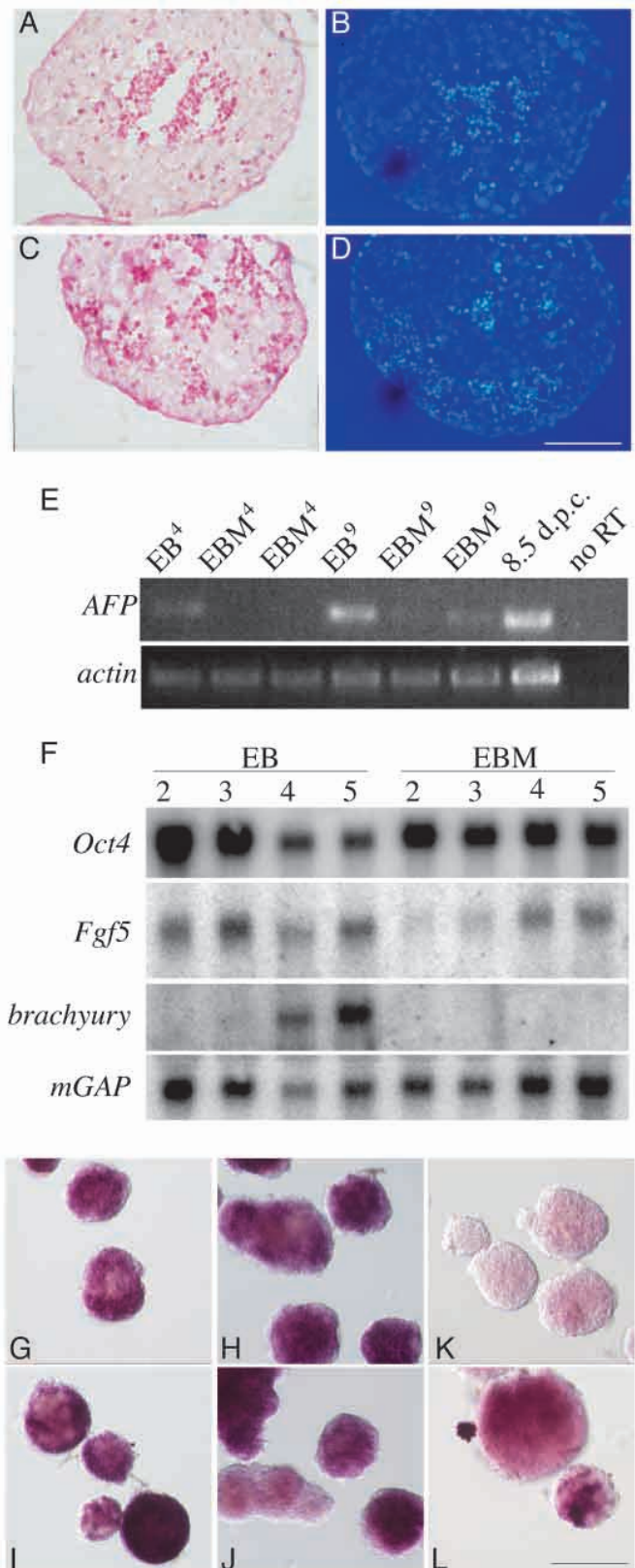
#### RNase protection analysis

20  $\mu\text{g}$  of cytoplasmic RNA, isolated from cellular aggregates (Rathjen et al., 2001), was analysed for the expression of *Sox1* and *mGAP* as described by Lake et al. (Lake et al., 2000).

#### In situ hybridisation analysis

In situ hybridisation of cell layers and whole-mount in situ hybridisation analysis of cell aggregates was performed using the method of Rosen and Beddington (Rosen and Beddington, 1993) with modifications (Rathjen et al., 1999; Lake et al., 2000). Antisense and sense probes for the detection of *Oct4*, *Fgf5* and *brachyury* were synthesised as described previously (Rathjen et al., 1999; Lake et al., 2000). Antisense *Sox1* probes were synthesised by T3 RNA polymerase as run-off transcripts from plasmid #1022 linearised with *Bam*HI. *Sox1* sense transcripts, used as controls, were obtained from the same plasmid linearised with *Hind*III and transcribed by T7 RNA

polymerase. *Sox2* transcripts were generated from a 748 bp *AccI/XbaI* cDNA fragment cloned into pBluescript SK. Transcripts were generated from *AccI* and *XbaI* linearised plasmid transcribed with T3 (antisense) and T7 (sense) RNA polymerases respectively. Both *Sox1*-



**Fig. 1.** Formation of early primitive ectoderm-like cells in suspension culture. (A-D). 7  $\mu\text{m}$  sections of EBM<sup>4</sup> (A,B) and EB<sup>4</sup> (C,D) stained with Haematoxylin and Eosin (A,C) and Hoechst 22358 (B,D) and viewed using brightfield (A,C) and fluorescent (UV2A filter; B,D) microscopy. Scale bar: 170  $\mu\text{m}$ . (E) RT-PCR analysis for the presence of AFP and actin transcripts in EB<sup>4</sup> and EB<sup>9</sup>, two independent populations of EBM<sup>4</sup> and EBM<sup>9</sup>, and 8.5 d.p.c. mouse embryos. A control reaction in which reverse transcriptase was omitted is included (no RT). (F) Northern blot analysis of 20  $\mu\text{g}$  of RNA isolated from EB<sup>2-5</sup> and EBM<sup>2-5</sup> probed for *Oct4* (1.55 kb), *Fgf5* (2.7 kb), *brachyury* (2.1 kb) and *mGAP* (1.5 kb). (G-L). Whole-mount in situ hybridisation analysis of EBM<sup>4</sup> (G,H,K) and EB<sup>4</sup> (I,J,L) probed with digoxigenin-labelled antisense probes to *Oct4* (G,I), *Fgf5* (H,J) and *brachyury* (K,L). Scale bar: 85  $\mu\text{m}$ .

and *Sox2*-containing plasmids were obtained from Dr R. Lovell-Badge, Division of Developmental Genetics, National Institute for Medical Research, Mill Hill, London. Digoxigenin-labelled *Gbx2* riboprobes were generated from pG290 which contains a 290 bp PCR fragment from base 780 to 1070 of the *Gbx2* cDNA (Chapman and Rathjen, 1995) cloned into pGEMT-easy (Promega). Antisense and sense probes were transcribed from *SalI* or *StyI* cut pG290, with T7 or T3 RNA polymerase, respectively. *Sox10* probes were transcribed from pSox10E.1 (obtained from Dr Peter Koopman, IMB, Brisbane, Australia). Anti-sense and sense probes were transcribed from *HindIII* or *BamHI* cut pSox10E.1 with T7 or T3 RNA polymerase, respectively.

Radiolabelled in situ hybridisation was performed as described previously (Keough et al., 1995). Antisense *Oct4* probe was synthesised by T3 RNA polymerase as run-off by transcripts from Bluescript containing a 462 bp *StuI Oct4* cDNA fragment (Schöler et al., 1990) linearised with *HindIII*.

#### PCR analysis of neuroectoderm gene expression

Total RNA was extracted from cell aggregates as described by Rathjen et al. (Rathjen et al., 2001). cDNA was synthesised from 1 µg of total RNA using Superscript™ II First-Strand Synthesis System for RT-PCR (Gibco BRL) following the manufacturer's instructions. PCR was performed using Platinum PCR Supermix (Gibco BRL) following the manufacturer's instructions. Reactions were performed in a capillary thermocycler (Corbett Research), with cycling parameters as follows; denaturing 94°C, 10 seconds, annealing 55°C, 10 seconds and extension 72°C, 60 seconds. Cycling times were determined for each primer set to be within the exponential phase of amplification. Primers for amplification of *actin*, *En-1*, *Hoxa7* and *Otx1* have been described previously (Okabe et al., 1996). Primer sequences and the length of amplified products were as follows:

*AFP* (471 bp)  
(5' CAAAGCATTGCACGAAAATG 3'; 5' TAAACACCCATCG-CACAGAT 3'),  
*Emx2* (198 bp)  
(5' CCAAAGCGGATTTCGAACCGC 3'; 5' TGAGCCTTCTCCT-CTAGC 3'),  
*En2* (512 bp)  
(5' AGGCTCAAGGCTGAGTTTCA 3'; 5' CAGTCCCCTTTCG-AGAAAAA 3'),  
*Hex1* (310 bp)  
(5' GGGAAGGTGCTCAGCTC 3'; 5' CGTCCTCGGTACCAAC-TC 3'),  
*HoxB1* (501 bp)  
(5' CGAAAGGTTGTAGGGCAAGA 3'; 5' CGGTCTGCTCAG-TTCCGTAT 3'),  
*Krox20* (502 bp)  
(5' GGAGGGCAAAGGAGATACC 3'; 5' GGTCCAGTTCAG-GCTGAGTC 3'),  
*Mash1* (482 bp)  
(5' CGTCCTCTCCGGAAGTACTGAT 3'; 5' TCCTGCTTCCAAA-GTCCATT 3'),  
*Nkx2.2* (514 bp)  
(5' CTCTTCTCCAAAGCGCAGAC 3'; 5' AACAACCGTGGTA-AGGATCG 3'),  
*Pax3* (502 bp)  
(5' CGTGTCAGATCCCAGTAGCA 3'; 5' CCTCCAGGAGGA-ACTACCC 3'),  
*Pax6* (500 bp)  
(5' AGTTCTTCGCAACCTGGCTA 3'; 5' TGAAGCTGCTGCTG-ATAGGA 3'),  
*Shh* (502 bp)  
(5' GGAACTCACCCCAATTACA 3'; 5' GAAGGTGAGGAAG-TCGCTGT 3').

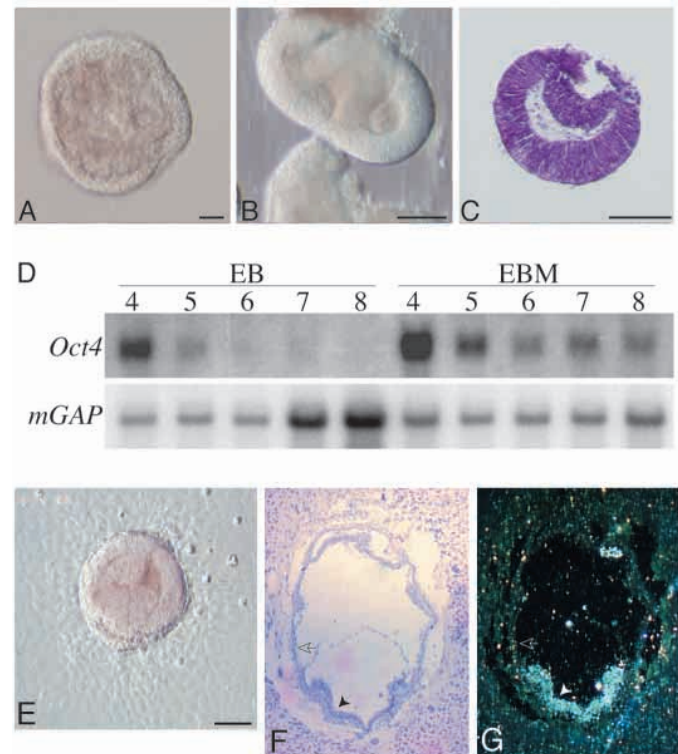
PCR products were analysed on 2% agarose gels and visualised with ethidium bromide.

#### Histological analysis

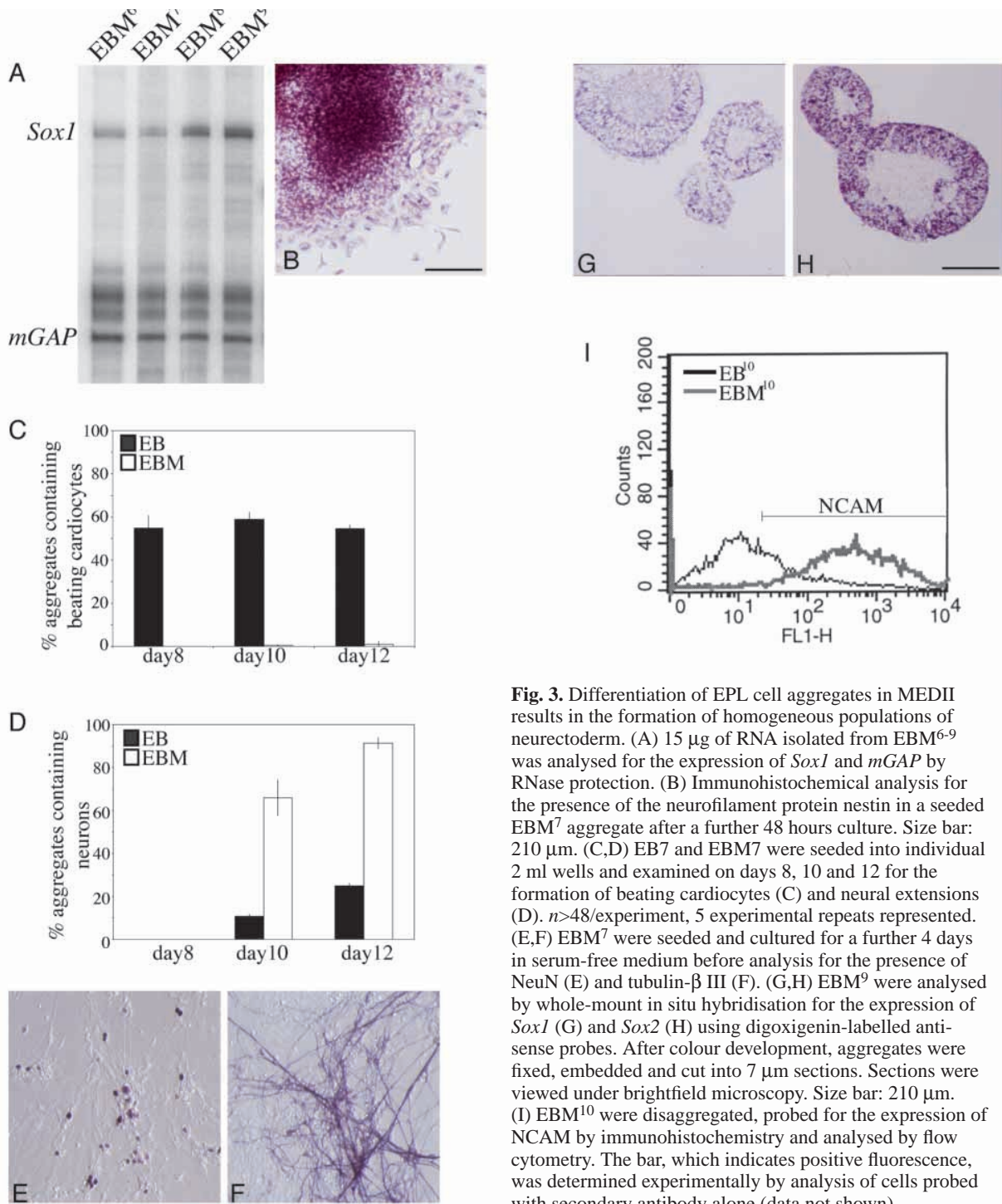
EB<sup>4</sup> and EBM<sup>4</sup> were fixed with 4% PFA for 30 minutes before embedding in paraffin wax and sectioning as described previously (Hogan et al., 1994). 7 µm sections were stained with Haematoxylin and Eosin (Kaufman, 1992), or with Hoechst 22358 (5 µg/ml in PBS; Sigma) for 5 minutes. EBMs, which had been analysed by whole-mount in situ hybridisation staining, were fixed in 4% PFA overnight, washed several times with PBS, 0.1% Tween 20, treated with 100% methanol for 5 minutes and then isopropanol for 10 minutes. Bodies were then treated and embedded as described previously (Hogan et al., 1994).

#### Immunohistochemical analysis

Cellular aggregates were fixed in 4% PFA in PBS for 30 minutes and dehydrated in sequential 30-minute washes in 50% ethanol and 70% ethanol. Cells were rehydrated in PBS and permeabilised with RIPA buffer (150 mM NaCl, 1% NP-40; 0.5% NaDOC, 0.1% SDS) for 30 minutes, washed in PBS and blocked in 10% goat serum, 2% BSA in PBS for 30 minutes. Primary antibodies, diluted in blocking buffer, were added and incubated overnight at 4°C. After washing in PBS, aggregates were incubated with alkaline phosphatase-conjugated, species-specific secondary antibodies directed against the primary



**Fig. 2.** Morphology and gene expression in differentiating pluripotent cells in vitro and in vivo. (A) EBM<sup>7</sup> and (B) EBM<sup>9</sup> viewed using Hoffmann interference contrast microscopy. (C) 7 µm section of an EBM<sup>9</sup> aggregate stained with haematoxylin and viewed using Hoffmann interference contrast microscopy. Size bars: 210 µm. (D) Northern blot analysis of 20 µg RNA isolated from EB<sup>4-8</sup> and EBM<sup>4-8</sup> probed for *Oct4* and *mGAP*. (E) Whole-mount in situ hybridisation analysis of an EBM<sup>7</sup> aggregate seeded and cultured for a further 24 hours and probed with a digoxigenin-labelled antisense probe to *Oct4*. Size bar: 210 µm. (F, G) 10 µm transverse section of a 7.75 d.p.c. mouse embryo probed with a radiolabelled antisense probe to *Oct4* viewed in brightfield (F) and darkfield (G) illumination. A concentration of silver grains can be seen over the neural ectoderm (black arrow). Mesoderm (outlined arrow).



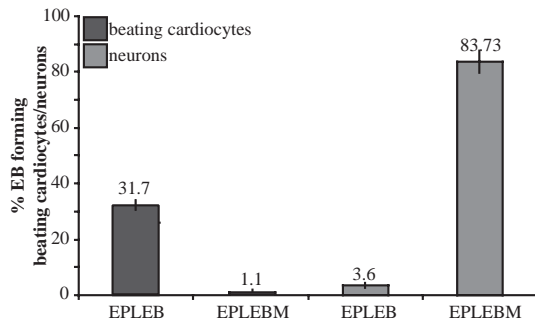
**Fig. 3.** Differentiation of EPL cell aggregates in MEDII results in the formation of homogeneous populations of neuroectoderm. (A) 15  $\mu$ g of RNA isolated from EBM<sup>6-9</sup> was analysed for the expression of *Sox1* and *mGAP* by RNase protection. (B) Immunohistochemical analysis for the presence of the neurofilament protein nestin in a seeded EBM<sup>7</sup> aggregate after a further 48 hours culture. Size bar: 210  $\mu$ m. (C,D) EB<sup>7</sup> and EBM<sup>7</sup> were seeded into individual 2 ml wells and examined on days 8, 10 and 12 for the formation of beating cardiocytes (C) and neural extensions (D).  $n > 48$ /experiment, 5 experimental repeats represented. (E,F) EBM<sup>7</sup> were seeded and cultured for a further 4 days in serum-free medium before analysis for the presence of NeuN (E) and tubulin- $\beta$  III (F). (G,H) EBM<sup>9</sup> were analysed by whole-mount in situ hybridisation for the expression of *Sox1* (G) and *Sox2* (H) using digoxigenin-labelled anti-sense probes. After colour development, aggregates were fixed, embedded and cut into 7  $\mu$ m sections. Sections were viewed under brightfield microscopy. Size bar: 210  $\mu$ m. (I) EBM<sup>10</sup> were disaggregated, probed for the expression of NCAM by immunohistochemistry and analysed by flow cytometry. The bar, which indicates positive fluorescence, was determined experimentally by analysis of cells probed with secondary antibody alone (data not shown).

antibodies in 100 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.5% blocking reagent (Boehringer Mannheim). Cellular aggregates were washed in Buffer 2 (100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 5 mM MgCl) and antibody conjugates were detected enzymatically with NBT and BCIP (both Boehringer Mannheim) made up in Buffer 2 according to the manufacturer's instructions. Aggregates were examined using a Nikon TE300 microscope with Hoffmann interference contrast optics. The antibodies used were directed against nestin (Developmental Studies Hybridoma Bank, reference Rat 401) used at a dilution of 1:150, tubulin- $\beta$  III (mouse anti-tubulin, beta II isoform; Chemicon #MAB1637) used at a dilution of 1:1000, NeuN

(mouse anti-neuronal nuclei, Chemicon #MAB377) used at a dilution of 1:200, and GFAP (anti-gial fibrillary acidic protein; Sigma #G9269) used at a dilution of 1/1000. Secondary antibodies were alkaline phosphatase-conjugated goat anti-mouse IgG (ZyMax grade, Zymed Laboratories Inc.) used at a concentration of 1:1000 and alkaline phosphatase-conjugated goat anti-rabbit IgG (ZyMax grade, Zymed Laboratories Inc.) used at a dilution of 1/1000.

#### Flow cytometry analysis

EB<sup>10</sup> and EBM<sup>10</sup> were collected and washed in PBS, then disaggregated by incubating for 5 minutes in 0.5 mM EDTA/PBS



**Fig. 4.** MEDII reduces the formation of mesoderm and promotes neuron formation from EPL cell embryoid bodies. EPLEB<sup>7</sup> and EPLEBM<sup>7</sup> aggregates were seeded into individual 2 ml wells and cultured for a further 5 days before scoring for the presence of beating cardiocytes and neural extensions.  $n > 48$ /experiment, 3 experimental repeats represented.

followed by vigorous pipetting and agitation to a single cell suspension. Cells were washed several times in PBS before fixation with 4% PFA for 30 minutes. Fixed cells were washed with 1% BSA/PBS, resuspended at  $1 \times 10^6$  cells/ml, and incubated with antibody directed against NCAM (Santa Cruz Biotech, SC-1507) at a dilution of 1:2 for 1 hour. Cells were washed with 1% BSA/PBS before incubation with FITC-conjugated goat anti-mouse IgM ( $\mu$ -specific; Sigma) used at a concentration of 1:100. FITC-conjugated goat anti-mouse IgM was pre-adsorbed for 1 hour in 1% BSA/PBS before use. Cells were washed in PBS and fixed in 1% PFA for 30 minutes. Data was collected on  $1 \times 10^4$  cells on a Becton Dickinson FACScan and analysis performed using CellQuest 3.1.

## RESULTS

### Formation of EPL cells from ES cells in suspension

Previous results described the formation of EPL cells from ES cells cultured in monolayer (Rathjen et al., 1999). To test the effects of suspension culture, ES cells were aggregated in IC:DMEM or IC:DMEM supplemented with 50% MEDII to form EBs and EBMs respectively. After 4 days, cellular aggregates formed in the presence of MEDII (EBM<sup>4</sup>) could be distinguished from EB<sup>4</sup> by morphology. Histological analysis of sectioned EB<sup>4</sup> and EBM<sup>4</sup> showed EBM<sup>4</sup> to comprise a multi-cell layer of uniform thickness surrounding a single, internal area of cell death indicated by the presence of pyknotic nuclei (Fig. 1A,B). In contrast, EB<sup>4</sup> were internally disorganised with sporadic, multiple foci of cell death dispersed throughout the aggregates (Fig. 1C,D). Consistent with the results of others (Doetschman et al., 1985) a morphologically distinct outer layer of extraembryonic endoderm was apparent at low levels in EB<sup>4</sup> and at higher levels in more advanced EBs, and expression of AFP, a marker of visceral endoderm, was detected in both EB<sup>4</sup> and EB<sup>9</sup> (Fig. 1E). By contrast, extra-embryonic endoderm could not be seen in EBM<sup>4</sup> or in later populations of EBMs. Furthermore, AFP expression could not be detected by RT-PCR in populations of EBM<sup>4</sup> and was detected only at extremely low levels in populations of EBM<sup>9</sup> (Fig. 1E), suggesting an absence of extra-embryonic cell types.

EB<sup>2-5</sup> and EBM<sup>2-5</sup> were analysed by northern blot (Fig. 1F) for the expression of *Oct4*, a marker gene for pluripotent cells

(Rosner et al., 1990; Schöler et al., 1990), and *Fgf5*, a gene up-regulated in pluripotent cells upon primitive ectoderm formation (Haub and Goldfarb, 1991). *Oct4* expression was maintained at high levels throughout these early stages of EBM development indicating that pluripotent cell differentiation had not commenced within these aggregates. High level *Oct4* expression in EBM<sup>4</sup> was accompanied by elevated *Fgf5* expression, indicating that the pluripotent cells had formed primitive ectoderm. Consistent with this, expression of *Rex1*, a marker of the pluripotent cells of the ICM but not primitive ectoderm, was down regulated between days 1 and 2 of EBM development (data not shown). In contrast, highest levels of *Oct4* and *Fgf5* expression in EBs were observed at days 2-3 and day 3 respectively. Downregulation of both genes in EB<sup>4</sup> indicated that pluripotent cells within these aggregates had commenced differentiation.

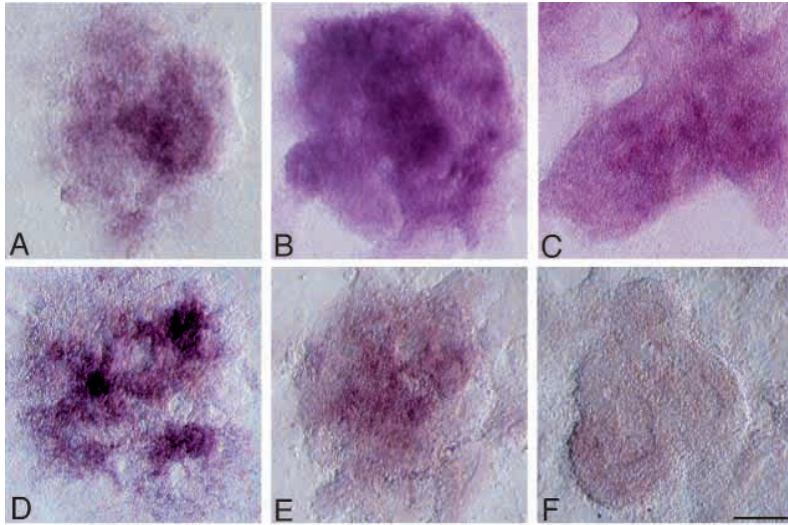
The distribution of pluripotent cells within aggregates was investigated by whole-mount in situ hybridisation of EB<sup>4</sup> and EBM<sup>4</sup> with *Oct4* and *Fgf5* antisense probes. Uniform expression of *Oct4* (Fig. 1G) and *Fgf5* (Fig. 1H) within and between individual EBM<sup>4</sup> aggregates was consistent with the deduced cellular homogeneity of primitive ectoderm within these aggregates and persistence of pluripotent cells to day 4. This contrasted with patchy expression of these markers within and between individual EB<sup>4</sup> aggregates (Fig. 1I,J), consistent with the variable onset and progression of pluripotent cell differentiation within EBs described here and by others (Haub and Goldfarb, 1991).

The expression of *brachyury*, a marker for nascent mesoderm (Herrmann, 1991), was used to confirm the onset of mesodermal differentiation in the aggregates. *Brachyury* expression was analysed in EBM<sup>2-4</sup> and EB<sup>2-4</sup> by northern blot (Fig. 1F) and in EBM<sup>4</sup> and EB<sup>4</sup> by whole-mount in situ hybridisation (Fig. 1K,L). In EBs, *brachyury* expression was up regulated on day 4 of development, coincident with the loss of pluripotency in the aggregates. In contrast *brachyury* expression could not be detected by either method in EBM<sup>2-4</sup>, consistent with the maintenance of *Oct4* expression and supporting a lack of differentiation within these aggregates. EBM<sup>4</sup> therefore appear to constitute a homogeneous population of EPL cells equivalent to embryonic primitive ectoderm.

MEDII has been shown to contain 50-100 units of human LIF (Rathjen et al., 1999). LIF has been shown to retard the developmental progression of EBs in vitro (Shen and Leder, 1992). ES cells aggregated and maintained in medium supplemented with 100 units of LIF did not duplicate the morphology or gene expression profile of EBMs (data not shown), indicating the importance of additional secreted factors in MEDII (Rathjen et al., 1999) for EPL cell induction.

### Directed formation of ectodermal and neuroectodermal lineages by EPL cell formation and differentiation

Continued culture of EBMs in medium containing 50% MEDII resulted in the formation of cellular aggregates displaying an unusual and distinct morphology. By day 7, >95% of the cellular aggregates within the EBM population had formed a convoluted stratified epithelial sheet of cells as shown in Fig. 2A. EBM<sup>7</sup> transferred to 50% DMEM:50% Hams F12 supplemented with ITSS and 10 ng/ml FGF2 for a further 2



**Fig. 5.** Temporal expression of *Sox1* and *Gbx2* in EPL cell-derived neuroectoderm. (A-F) Seeded EBM<sup>7</sup> were cultured for a further 24 (A,D), 48 (B,E) and 72 (C,F) hours and analysed by in situ hybridisation with digoxigenin-labelled antisense probes directed against *Sox1* (A-C) and *Gbx2* (D-F). Aggregates were viewed using Hoffmann interference contrast microscopy. Size bar: 210  $\mu$ m.

days of culture, formed a population in which the cells of the monolayer appeared to have become more columnar (Fig. 2B,C). Populations of EBM<sup>9</sup> were relatively homogeneous, with >95% of the aggregates exhibiting this distinctive morphology, and the differentiation was reproduced routinely using both the D3 and E14 cell lines. Cellular aggregates of similar morphology were not detected within the EB<sup>7</sup> or EB<sup>9</sup> populations although equivalent cell layers could be detected within a proportion of individual aggregates (data not shown). Cell death was not observed during the further differentiation of EBM<sup>4</sup> suggesting that this morphological homogeneity was achieved by directed differentiation and not formation and subsequent loss of other cell lineages. This is in contrast to many other published methodologies in which the requirement for selection or use of toxic chemicals results in significant cell death (J. R., unpublished) (Tropepe et al., 2001).

Northern blot analysis of EB<sup>4-8</sup> and EBM<sup>4-8</sup> showed a down-regulation of *Oct4* in both populations (Fig. 2D) indicating differentiation of the pluripotent cells within both populations. However, while *Oct4* was undetectable in EBs after day 5, a low but consistent level of *Oct4* expression, 4.2-fold lower than EBM<sup>4</sup>, was detected in EBMs on all days of development after day 5. EBM<sup>7</sup> were seeded and analysed after a further 24 hours culture (EBM<sup>8</sup>) by whole-mount in situ hybridisation with a DIG-labelled *Oct4* antisense probe. This analysis failed to detect cells expressing *Oct4* at levels equivalent to pluripotent cells (Fig. 2E). Similarly, in situ hybridisation analysis of a single cell suspension of EBM<sup>9</sup> failed to detect *Oct4*-expressing cells (data not shown), suggesting that the *Oct4* expression detected by northern blot analysis represented low level expression by the majority of cells within the population and not expression by a small population of residual pluripotent cells within the aggregates. Previous reports (Rosner et al., 1990; Schöler et al., 1990) and data shown here (Fig. 2F,G) demonstrate maintenance of *Oct4* expression after gastrulation in the neuroectoderm lineage at 7.75-8.5 d.p.c. In conjunction with the overt neuroectoderm morphology (Fig. 2B,C), low level expression of *Oct4* suggested formation of neuroectoderm within EBMs.

RNA from EBM<sup>6-9</sup> was analysed by RNase protection for the expression of *Sox1* (Fig. 3A), a marker which has been shown to delineate the neural plate and is expressed by all

undifferentiated neural cells (Pevny et al., 1998). Furthermore, EBM<sup>7</sup> seeded for 48 hours were analysed by immunohistochemistry for expression of nestin, a neurofilament protein expressed in neural progenitor cells (Fig. 3B) (Zimmerman et al., 1994). Expression of both *Sox1* and nestin by EBMs indicated the formation of neuroectoderm. Consistent with earlier results (Fig. 1), the expression of *brachyury* was not detected within these later EBM populations by in situ hybridisation (data not shown).

Spontaneously differentiated cells were apparent in and surrounding aggregates after seeding. Individual EBM<sup>7</sup> and EB<sup>7</sup> were seeded and assessed on days 8, 10 and 12 (Fig. 3C,D) for the presence of beating cardiocytes, a differentiated mesoderm derivative, and neurons, a differentiated ectoderm derivative that were identified by morphology and the expression of the markers neurofilament 200 (data not shown), neuronal nuclei (NeuN) (Mullen et al., 1992) and the  $\beta$  III isoform of tubulin (Draberova et al., 1998) (Fig. 3E,F). Consistent with the up-regulation of neuroectoderm-specific markers, and lack of *brachyury* expression, neurons were formed in the majority of EBMs (91.33%) while <2% of EBMs formed beating cardiocytes. In contrast, as expected for heterogeneous differentiation, EBs contained a mixed population of differentiated cells that included both beating cardiocytes (54.5%) and neurons (24.9%) on day 12.

### EBMs constitute a homogeneous population of neural progenitor cells

Morphology and differentiation of EBMs suggested that within the population nearly 100% of the cellular aggregates were neural progenitor cells. The number of cells within the population expressing neural-specific markers was evaluated to assess the homogeneity of differentiation. EBM<sup>9</sup> were probed by whole-mount in situ hybridisation for expression of *Sox1*, and *Sox2*, which shows a similar expression pattern to *Sox1* but is expressed earlier in embryogenesis (Pevny et al., 1998). Representative sections (Fig. 3G,H) showed that EBM<sup>9</sup> was a morphologically uniform population of cells equivalent to the neuroectoderm-like monolayer, in which each cell stained positive for expression of *Sox1* and *Sox2*. No signal was detected with sense probes (data not shown).

To enable comparative quantitation of neuroectoderm formation, EBM<sup>10</sup> and EB<sup>10</sup> were disaggregated to a single cell suspension, labelled immunocytochemically with antibodies directed against NCAM, a cell adhesion molecule expressed strongly in the nervous system (Rutishauser, 1992; Ronn et al., 1998), and analysed by flow cytometry (Fig. 3I). 95.7% of cells from EBM<sup>10</sup> were scored positive for NCAM expression, demonstrating relatively uniform differentiation of these aggregates to neural lineages. In comparison, only 42.13% of

cells from EB<sup>10</sup> expressed NCAM, consistent with the established heterogeneity of ES cell differentiation within this system.

### MEDII redirects EPL cell differentiation from mesoderm to ectoderm

It has been previously reported that EPL cells form neurons poorly, if at all, when differentiated as EBs, but form elevated levels of nascent and differentiated mesoderm (Lake et al., 2000). This has been interpreted as reflecting disrupted signalling from visceral endoderm or visceral endoderm-derived ECM (Lake et al., 2000; Rathjen et al., 2001). EPL cells, formed by culture of ES cells in IC:DMEM supplemented with 50% MEDII for 2 days, were aggregated and cultured in suspension for 7 days in either IC:DMEM (EPLEBs) or IC:DMEM supplemented with 50% MEDII (EPLEBM). On day 7, individual EPL cell embryoid bodies were seeded onto gelatin-treated tissue culture plasticware in IC:DMEM. On day 8 the medium was changed to DMEM:F12 and embryoid bodies were cultured for a further 4 days before microscopic inspection for the presence of beating cardiocytes and neurons.

As shown in Fig. 4A, EPL cell embryoid bodies formed beating cardiocytes efficiently (35.25%) but neurons at low levels (3.6%), consistent with previous reports and gene expression (Lake et al., 2000). In contrast, EPL cell embryoid bodies cultured in the presence of 50% MEDII (EPLEBM) exhibited significantly lower levels of beating cardiocyte formation (0.9%), and an up regulation in neuron formation to 83.73% (Fig. 4B). These data suggest that signals contained within MEDII replace those deficient in the EPLEB differentiation environment (Lake et al., 2000; Rathjen et al., 2001) to direct the pluripotent cells to an ectodermal/neural fate.

### Neural formation within EBMs is relatively synchronous and reflects the temporal formation of neural lineages in the embryo

During embryogenesis, formation of neurectoderm is characterised by progressive alterations in gene expression. The neural plate, which contains the earliest neural precursors, is characterised by expression of *Sox1* within a group of cells on the anterior midline of the embryo (Pevny et al., 1998). This population of cells also expresses the homeobox gene *Gbx2* (Wassarman et al., 1997). With continued development the neural plate folds at the midline and the outer edges close to form the neural tube. *Sox1* expression is maintained after tube closure but *Gbx2* expression is down regulated in the majority of cells of the neural lineage and persists only in a restricted population of cells at the mid-brain/hind-brain boundary (Wassarman et al., 1997).

After further culture for 24, 48 and 72 hours, whole-mount in situ hybridisation of seeded EBM<sup>7</sup> was used to investigate the temporal regulation of *Sox1* and *Gbx2* during EBM progression. After 24 hours, *Sox1* was expressed in approximately 50% of the cells within the seeded aggregates (Fig. 5A). The extent of *Sox1* expression was increased after 48 hours, and evident in the majority of cells within the aggregates after 48 and 72 hours culture (Fig. 5B,C), indicating formation of neurectoderm. *Gbx2* was also expressed in approximately 50% of the cells within the seeded aggregates

after 24 hours, but was seen in fewer cells within the population in after 48 hours and was virtually undetectable after 72 hours (Fig. 5D,E,F). The loss of *Gbx2* expression in aggregates in which *Sox1* expression persists recapitulates the temporal regulation of this gene in the developing neural tube of the embryo and suggests that the progression of neurectoderm formation in vitro recapitulates the formation of this cell population in vivo.

### EPL cell-derived neurectoderm induced by MEDII is not positionally specified

In vivo the neural tube acquires region-specific gene expression with respect to both the rostral-caudal and dorsal-ventral axes, indicative of restricted developmental fate. Expression of markers of the neural tube in vivo shortly after closure in restricted anterior, posterior and ventral domains was analysed in EBM<sup>9</sup> by RT-PCR, and compared to EB<sup>9</sup>, which is a mixed population of cells containing ectoderm and mesoderm, and EPLEB<sup>9</sup>, a mesoderm-enriched, ectoderm deficient (Lake et al., 2000) population.

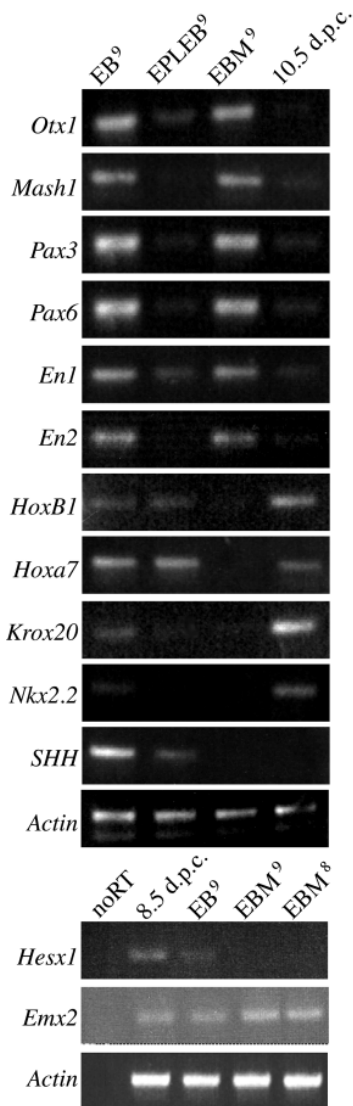
As shown in Fig. 6, the expression of genes marking presumptive forebrain, *Hesx1* (Thomas and Beddington, 1996) and *Nkx2.2* (Price et al., 1992), individual rhombomeres of the hindbrain, *HoxB1* (Studer et al., 1998) and *Krox20* (Nieto et al., 1991), posterior ectoderm and trunk, *Hoxa7* (Mahon et al., 1988) and ventral neural tube, *Shh* (Marti et al., 1995) was not detected in EBM<sup>9</sup>. Furthermore, the absence of *Shh* expression suggests that the signalling pathways leading to ventralisation of the neural tube were not active in the EBM system (Echelard et al., 1993).

*En1*, *En2* and *Otx1* are expressed in a broad region of the anterior neural tube around the time of closure and subsequently within defined regions of the midbrain (Davis and Joyner, 1988; Simeone et al., 1998). These genes were expressed in EBM<sup>9</sup> as was *Mash1*, a gene expressed in domains of the neuroepithelium of the forebrain, midbrain and spinal cord between days 8.5 and 10.5 d.p.c. (Guillemot and Joyner, 1993), *Pax3* and *Pax6* (Goulding et al., 1991; Walther and Bruce, 1991), and *Emx2*, a gene expressed in the forebrain at 8.5 d.p.c. (Simeone et al., 1992). Gene expression therefore suggested that neurectoderm formed within EBMs lacked positional information and was most similar to unspecified anterior neurectoderm with characteristics of fore- and mid-brain.

Consistent with the described mesodermal differentiation within EPLEBs (Lake et al., 2000), expression of neural marker genes in EPLEB<sup>9</sup> was absent or detected at very low levels. Where expression was detected it was ascribed to additional, non-neural sites of expression in the embryo, for example, *Shh* expression in the prechordal plate (Marti et al., 1995), *Hoxb1* expression in primitive streak mesoderm (Studer et al., 1998), *Hoxa7* expression in the primordia of the vertebrae and ribs (Mahon et al., 1988), *Pax3* expression in newly formed somites and later in the dermomyotome (Goulding et al., 1991) and *En1* expression in tissues of somitic origin (Davis and Joyner, 1988).

Expression of all genes was detected in EB<sup>9</sup>, reflecting the complex mix of cell populations formed in this differentiation environment. As for EPLEBs, a proportion of this expression could be attributed to non-neural sites of embryonic expression. However, the expression within EB<sup>9</sup> of *Krox20* and





**Fig. 6.** Expression of neurectoderm markers in EPL cell-derived neurectoderm. cDNA was synthesised from 1  $\mu$ g of total RNA isolated from EB<sup>9</sup>, EPLEB<sup>9</sup>, EBM<sup>9</sup> and 10.5 d.p.c. mouse embryo (used as a positive control), or EB<sup>9</sup>, EBM<sup>8</sup>, EBM<sup>9</sup> and 8.5 d.p.c. mouse embryo (used as a positive control), and used as a template for PCR analysis of the genes denoted. Expression of *actin* was used as an example of a gene expressed in all cell types to normalise the PCR reaction. Primer sequences and product sizes can be found in the Materials and Methods.

*Nkx2.2*, the expression of which is restricted to limited domains within the neural lineage, indicated that cryptic positional information is generated within the EB environment.

### EPL cell-derived neurectoderm has a developmental potential consistent with embryonic neural tube and can be directed to neural crest or glial lineages in response to exogenous signals

Embryonic neurectoderm acts as the progenitor population for the neural, glial and neural crest lineages *in vivo*. Others have developed conditions that promote formation of the neural crest and glial lineages from neural precursors *in vitro*. Neural tube explants from the quail have been shown to form neural crest in response to the protein kinase C inhibitor staurosporine (Newgreen and Minichiello, 1996). EBM<sup>9</sup> were dissociated to clumps and cultured in medium supplemented with either 25 nM staurosporine in DMSO or 0.1% DMSO. Within 3 hours of seeding into medium containing staurosporine, EBMs explants were surrounded by a halo of morphologically uniform migrating cells (Fig. 7A), which appeared indistinguishable from avian neural crest cells produced from avian neural tube in response to staurosporine (Newgreen and

Minichiello, 1996). The phenotypic alteration induced by staurosporine was homogeneous across the population of aggregate explants, and was not observed in EBM explants cultured in medium containing 0.1% DMSO (Fig. 7B). This differentiation was observed in the presence of 1 nM to 100 nM staurosporine, although efficient, homogeneous differentiation required concentrations of 10 nM or greater (data not shown). After 48 hours culture, EBM explants were analysed by *in situ* hybridisation for expression of *Sox10* (Fig. 7D) which is up regulated on the formation of mouse neural crest *in vivo* (Southard-Smith et al., 1998). Consistent with the crest-like morphology of the differentiating cells, *Sox10* expression was observed in all migratory cells cultured in medium containing staurosporine, but not in cells cultured in medium containing 0.1% DMSO.

Sequential culture of ES cell derived neural stem cells in EGF/laminin and PDGF-AA has been shown to enrich for glial lineages (Brustle et al., 1999). EBM<sup>9</sup> explants were cultured in medium containing FGF2 (10 ng/ml), EGF (20 ng/ml) and laminin (1  $\mu$ g/ml). After 5 days EGF and laminin were omitted from the medium and PDGF-AA was added to a concentration of 10 ng/ml for a further 2-3 days. Cells were not trypsinised or triturated during differentiation. Differentiation of EBM<sup>9</sup> explants in response to EGF/laminin and PDGF-AA followed a homogeneous morphological progression depicted in Fig. 7E-G. After PDGF-AA treatment cultures were analysed by immunohistochemistry for the expression of glial fibrillary acidic protein (GFAP), a marker expressed by both glial precursors and differentiated astrocytes (Landry et al., 1990). Consistent with the uniform morphology of the cells formed, >95% of differentiated cells formed from EBM explants, using this protocol, expressed GFAP (Fig. 7H), indicating homogeneous differentiation of EPL cell-derived neurectoderm to cells of the glial lineage.

EPL cell-derived neurectoderm therefore forms a range of cell types, including neurons, and responds to exogenous signals in a manner consistent with the known properties of embryonic neurectoderm. Homogeneous formation of differentiated products, in contrast to that described elsewhere (Brustle et al., 1999), is indicative of homogeneity within the starting neurectoderm population.

## DISCUSSION

We have demonstrated here the formation, from pluripotent cells, of a homogeneous population of neural precursors equivalent to the embryonic neural epithelium, the neurectoderm. This cell type is normally found in the neural plate and neural tube. Unlike previously described methodologies, MEDII-directed differentiation recapitulates establishment of the neural lineage in the embryo, with the sequential elaboration of intermediate populations, and results in a homogeneous population of neurectoderm as characterised by morphology, gene expression and differentiation potential. Without the concurrent formation of alternative ES cell differentiation products, such as extraembryonic endoderm, differentiation occurs in an environment free of known sources of instructive signals, which permits single lineage differentiation. This results in formation of a naïve or unpatterned neurectoderm, a cell population previously

unidentified *in vivo* or *in vitro*. Synchronous and homogeneous formation of the embryonic neural precursor provides a powerful system for elucidating the molecular and cellular interactions required for formation and patterning of the neural lineage, and a well characterised neural precursor for implantation studies and further differentiation into homogeneous populations of terminally differentiated neural cell populations.

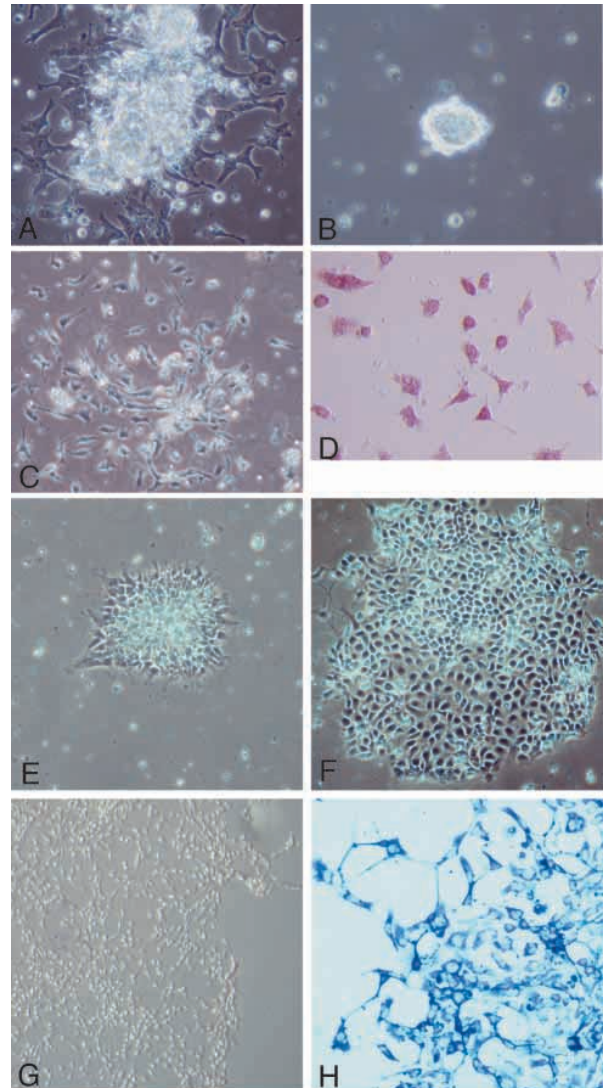
### Formation of EPL cells/primitive ectoderm in suspension culture

Aggregation of ES cells in medium supplemented with MEDII (EBMs), resulted in the homogeneous and synchronous formation of EPL cells from ES cells in suspension, a transition previously demonstrated only in adherent culture. On day 4 of development EBMs constituted a homogeneous population, which were characterised by morphology and the acquisition of a gene expression profile equivalent to EPL cells, with the expression of *Oct4* and *Fgf5*, but not *Rex1*. As expected these cells exhibited a broad differentiation potential, able to form both ectoderm and mesoderm (Fig. 3; data not shown). However no detectable associated differentiated cells, including cells of the primitive endoderm lineage, were seen in EBM<sup>4</sup>.

EBM<sup>4</sup> formed cellular aggregates of distinctive morphology with a homogeneous multiple cell layer encompassing a single region of cell death. Analysis of EBMs earlier in development did not show formation of multiple foci of cell death that merged to form the single foci seen in EBM<sup>4</sup>. This is in contrast to EBs, which have been shown here and by others to form multiple foci of cell death at early stages that combine to form a single cavity (Coucounanis and Martin, 1995). Cavity formation has been postulated to result from the activity of two distinct signals within the embryoid body, a diffusible 'death' signal from the extraembryonic endoderm and a matrix-associated survival signal from the extracellular matrix formed between the endoderm and pluripotent cells (Coucounanis and Martin, 1995). EBMs, however, did not form the extraembryonic endoderm lineage, as assessed by morphology and gene expression and would as a consequence lack the 'death' signal. Similarly, cavitation and formation of a columnar primitive ectoderm epithelium in the absence of extraembryonic endoderm has been observed in EBs cultured in medium supplemented with ECM proteins (Li et al., 2001). These experiments question the requirement for a death signal in EB cavitation and support an alternative model for induction of apoptosis in pluripotent cells within EBs, perhaps from loss of cell-ECM contact (Li et al., 2001).

### Programmed differentiation of pluripotent cells to neuroectoderm *in vitro*

Differentiation of EPL cells as aggregates in medium without MEDII (EPLBs) results in the efficient formation of mesoderm with an accompanying failure to form neurons (Lake et al., 2000). Furthermore, gene expression analysis of EPLBs differentiation did not detect expression of the ectoderm-specific gene *Sox1*, suggesting that differentiation within this system led to the preferential formation of mesoderm. Gene expression and differentiation analyses indicated that continued culture of EBM<sup>4</sup>, which formed an homogeneous population of EPL cells, in the presence of



**Fig. 7.** EPL cell-derived neuroectoderm can be directed to neural crest and glial lineages (A-D). EBM<sup>9</sup> explants were seeded onto cellular fibronectin-treated tissue culture plasticware in medium supplemented with 25 nM staurosporine/0.1% DMSO (A,C,D) or 0.1% DMSO alone (B). Cultures were examined after 3 (A,B) or 48 hours (C,D). (D) *In situ* hybridisation analysis of EBM<sup>9</sup> explants with digoxigenin-labelled antisense probes for *Sox10*. (E-H) EBM<sup>9</sup> explants were seeded onto poly-L-ornithine-treated tissue culture plasticware in medium supplemented with 10 ng/ml FGF2, 20 ng/ml EGF and 1 µg/ml laminin (E,F) followed by culture in medium supplemented with 10 ng/ml PDGF-AA (G,H). Cultures were examined after 2 (A), 4 (F) and 6 (G,H) days. (H) Immunohistochemistry of EBM<sup>9</sup> explants with antibodies directed against glial fibrillary acidic protein (GFAP).

MEDII, programs differentiation of the pluripotent cells to a relatively homogeneous population of neuroectoderm in the absence of extraembryonic endoderm lineages or other germ lineages. Consistent with this, EPL cell-derived neuroectoderm failed to express positional markers induced by visceral endoderm (*Hex1*) and notochord (*Shh*) (Echalard et al., 1993; Thomas and Beddington, 1996). Differentiation in response to MEDII was complete, without residual pluripotent cells detectable within the cellular aggregates. Cells within these

aggregates were organised as a stratified neural epithelium, morphologically equivalent to the neural epithelium established during neural induction in embryogenesis. This contrasts with previous reports of production of neural precursors from ES cells which do not result in organisation of cells into a neural epithelium (Bain et al., 1996; Okabe et al., 1996; Li et al., 1998; Kawasaki et al., 2000; Tropepe et al., 2001). Supplementation of EPLEB culture medium with MEDII led to a reduction in mesoderm formation and redirection of pluripotent cells to a neurectodermal cell fate. These data suggest that activities within the conditioned MEDII direct the differentiation of pluripotent cells to the neurectodermal lineage.

Induction of the neural lineage in lower vertebrates has been suggested to occur in response to BMP4 antagonists such as noggin and chordin emanating from Spemann's organiser (reviewed by Streit and Stern, 1999). A site of equivalent organiser activity has been demonstrated to occur at the time of gastrulation in birds and mammals, called Henson's node and node respectively (reviewed by Smith and Schoenwolf, 1998). However, increasing evidence suggests that these organiser structures in higher vertebrates do not play an equivalent role in neural induction. Ablation of *HNF3 $\beta$*  in mice results in embryos lacking a morphological node, node gene expression and node derivatives. However, these embryos undergo both neural induction and some neural patterning (Klingensmith et al., 1999). Similarly, mice lacking the organiser-specific gene *gooseoid*, misexpression of which results in formation of a supernumerary axis in *Xenopus* (Cho et al., 1991; Blum et al., 1992), manifest no obvious defects in gastrulation or neural induction (Yamada et al., 1995; Rivera-Perez et al., 1995). Consistent with this, misexpression of BMP4 antagonists in chick and mouse failed to demonstrate a relationship between BMP4 antagonism and neural induction (Streit et al., 1998; Klingensmith et al., 1999; Streit et al., 2000).

The programmed lineage-specific differentiation of pluripotent cells described here relies on initial formation of EPL cells from ES cells, and the activity of biologically derived factors found within the conditioned medium MEDII. This results in the sequential and relatively synchronous formation of progressively more differentiated intermediate cell populations with a temporal progression equivalent to embryogenesis. Sequential alteration of the differentiation environment can be used to direct differentiation of the neural progenitor cells to alternate neural fates. The inductive factors in MEDII required for ectodermal and neurectodermal formation from pluripotent cells have not been characterised. Previous demonstration that EPL cells differentiated as EPLEBs fail to form both the neurectoderm lineage and the extraembryonic visceral endoderm lineage has been interpreted as evidence that neurectoderm induction requires visceral endoderm or visceral endoderm-associated signalling (Lake et al., 2000; Rathjen et al., 2001). This is in contrast to a recent report supporting a default mechanism of neural determination from pluripotent cells (Tropepe et al., 2001). However, the low efficiency of neural determination that occurred spontaneously from ES cells (0.2%) compared to the robust induction of neural differentiation seen here questions the relevance of this differentiation pathway. Liver cells and cell lines, including HepG2 cells, share similarities in gene expression with

extraembryonic visceral endoderm (Meehan et al., 1984; Rossant, 1995; Barbacci et al., 1999), therefore the induction of neurectoderm by MEDII may result from a recapitulation of visceral endoderm signalling (Rathjen et al., 2001). Fractionation of MEDII should establish the nature of the neural induction signal.

Paradoxically, MEDII can be used to maintain a population of EPL cells in adherent culture for several passages without induction of a neural cell fate within the cells (Rathjen et al., 1999; Lake et al., 2000), suggesting a role for maintenance of cell-cell contact and/or cell-ECM association in neurectoderm induction. During gastrulation, neurectoderm arises from pluripotent cells positioned in the antero-distal portion of the pregastrulation egg cylinder. With gastrulation and recruitment of pluripotent cells to the primitive streak, this population of cells expands and populates the anterior half of the egg cylinder (Quinlan et al., 1995). Throughout gastrulation cells fated to contribute to ectoderm lineages maintain cell-cell contact and contact with the ECM and do not delaminate or enter the primitive streak. In contrast, migration of cells through the primitive streak involves loss of cell-cell and cell-ECM interactions and results in establishment of the mesodermal lineages. *FgfR1*<sup>-/-</sup> pluripotent cells, which are unable to migrate through the primitive streak, accumulate on the border of the streak and form a second site of neurectoderm formation (Ciruna et al., 1997). Like cells of the anterior ectoderm, *FgfR1*<sup>-/-</sup> cells fail to delaminate and maintain cell-cell and cell-ECM contact during gastrulation suggesting that these environmental cues are involved in pluripotent cell differentiation and determination of neural cell fate during gastrulation. Purification of active components of MEDII has identified a known ECM component within the medium (Bettes, 2001) which may act to enforce ECM association of pluripotent cells during differentiation as EBM and act to suppress the epithelial to mesenchymal transition associated with mesoderm induction in vivo.

### EPL cell-derived neurectoderm lacks positional specification

Signals required for the expression of positionally restricted genes within neurectoderm have been postulated to originate from adjacent cell populations such as the notochord, overlying ectoderm and visceral/definitive endoderm (Echelard et al., 1993; Thomas and Beddington, 1996; Liem et al., 1997). As might be expected for neurectoderm formed in the absence of potentially interacting cell types, the expression of many positionally restricted genes, including markers for the forebrain, hindbrain and trunk, could not be detected. Furthermore, *Shh*, the product of which has been implicated in establishment of ventral specification, did not appear to be expressed in EPL cell-derived neurectoderm. However, the expression of a subset of genes broadly expressed within neurectoderm around the time of neural tube closure was detected in EBM<sup>9</sup>. Many of these genes are expressed within the midbrain and forebrain suggesting that ES cell-derived neurectoderm may represent a neural cell progenitor population with equivalence to anterior neurectoderm. Alternatively, this gene expression may be characteristic of nascent neurectoderm, with expression restricted with regionalisation of the neural tube. For example, although expression of *Pax3* and *Pax6* is restricted positionally to dorsal

and ventral aspects of the neural tube, respectively (Goulding et al., 1991; Walther and Gruss, 1991), evidence from chick (Goulding et al., 1993) suggests that both these genes are widely expressed at a low level in neural tube before their expression domains become restricted in response to ventral specification. Although expression of the forebrain marker *Emx2* has not been reported prior to 8.5 d.p.c., a similar situation could account for the expression of this gene in EBM<sup>9</sup>. This would suggest that EPL cell-derived neurectoderm represents an unspecified population of neural cell precursors.

Gene expression was much more promiscuous in EB<sup>9</sup>, with detection of all positionally restricted neural patterning genes analysed within this system. This indicates that stochastic differentiation within the EB system is accompanied by expression of cryptic positional specification. Cells formed within this complex environment could potentially be exposed to multiple and, in some cases, inappropriate signals.

Consistent with the postulation of EPL cell-derived neurectoderm as naïve, the developmental analysis of these cells demonstrated potential to form cells of the neural, glial and neural crest lineages. The homogeneity of differentiation observed with directed differentiation to glial and neural crest lineages suggested that no pre-existing commitment to cell fate was present within the starting population.

Ability to form a primitive ectoderm-like cell population without concomitant formation of the extraembryonic endoderm lineage allows the development of directed differentiation from pluripotent cells in response to exogenous signals. Homogeneity and synchrony of differentiation can be achieved as a consequence of the lack of endogenous signalling from the primitive/visceral endoderm and/or subsequently from contribution by alternative germ lineages. The directed differentiation of EPL cells to neurectoderm in response to MEDII appears to recapitulate the temporal progression of lineage specification observed during embryonic neurogenesis. This technology, combined with the ability to precisely manipulate the genome of ES cells, will allow generation of model systems for the investigation of inductive signalling pathways involved in cell differentiation and specification in embryogenesis. Furthermore, homogeneous and synchronous differentiation will allow the generation of populations enriched in differentiated and progenitor cell types with the developmental plasticity best suited to transplantation.

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